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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/AU96/00018 <b>(22) International Filing Date:</b> 16 January 1996 (16.01.96)  <b>(30) Priority Data:</b> PN 0589 16 January 1995 (16.01.95) AU PN 0590 16 January 1995 (16.01.95) AU  <b>(71) Applicant (for all designated States except US):</b> NORTHERN SYDNEY AREA HEALTH SERVICE [AU/AU]; Pacific Highway, St. Leonards, NSW 2065 (AU).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> MANOLIOS, Nicholas [AU/AU]; 244 Doncaster Avenue, Kensington, NSW 2033 (AU).  <b>(74) Agent:</b> F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2140 (AU).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NOVEL PEPTIDE		
<b>(57) Abstract</b>  The present invention provides peptides which affect T-cells, presumably by action on the T-cell antigen receptor. The present invention further relates to the therapy of various inflammatory and autoimmune disease states involving the use of these peptides. Specifically, the peptides are useful in the treatment of disorders where T-cells are involved or recruited. The peptide is of the following formula: A-B-C-D-E in which: A is absent or 1 or 2 hydrophobic amino acids, B is a positively charged amino acid, C is a peptide consisting of 3 to 5 hydrophobic amino acids, D is a positively charged amino acid, and E is absent or up to 8 hydrophobic amino acids.		

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### ***Novel Peptide***

The present invention relates to peptides which affect T-cells, presumably by action on the T-cell antigen receptor. The present invention  
5 further relates to the therapy of various inflammatory and autoimmune disease states involving the use of these peptides. Specifically, the peptides are useful in the treatment of disorders where T-cells are involved or recruited.

T-cells are a subgroup of cells which together with other immune  
10 cell types (polymorphonuclear, eosinophils, basophils, mast cells, B-, NK cells), constitute the cellular component of the immune system. Under physiological conditions T-cells function in immune surveillance and in the elimination of foreign antigen(s). However, under pathological conditions there is compelling evidence that T-cells play a major role in the causation  
15 and propagation of disease. In these disorders, breakdown of T-cell immunological tolerance, either central or peripheral, is a fundamental process in the causation of autoimmune disease.

Central tolerance involves thymic deletion of self reactive cells (negative selection) and positive selection of T-cells with low affinity for  
20 self major histocompatibility complex antigens (MHC). In contrast, there are four, non-mutually exclusive hypotheses that have been proposed to explain peripheral T-cell tolerance which are involved in the prevention of tissue specific autoimmune disease. These include: anergy (loss of co-stimulatory signals, down regulation of receptors critical for T-cell  
25 activation), deletion of reactive T-cells, ignorance of the antigen by the immune system and suppression of autoreactive T-cells. Tolerance once induced does not necessarily persist indefinitely. A breakdown in any of these mechanisms may lead to autoimmune disease.

Autoimmune disease and other T-cell mediated disorders are  
30 characterised by the recruitment of T-cells to sites of inflammation. T-cells at these sites, coupled with their ability to produce and regulate cytokines and influence B-cell function, orchestrate the immune response and shape the final clinical outcome. An understanding of the process of antigen recognition and subsequent T-cell activation, leading to T-cell proliferation  
35 and differentiation, is therefore pivotal to both health and disease. The critical component of antigen recognition on the surface of T-cells is the

complex antigen receptor (TCR) which is a multisubunit structure that recognises antigen in the context of MHC-encoded proteins on the surface of antigen-presenting cells. Disturbance in this intricate structure-function relationship of the TCR, integrating antigen recognition with T-cell  
 5 activation may provide the therapeutic means to deal with inflammation and T-cell mediated disorders.

The TCR is composed of at least seven transmembrane proteins. The disulfide-linked ( $\alpha\beta$ -Ti) heterodimer forms the clonotypic antigen recognition unit, while the invariant chains of CD3, consisting of  $\epsilon$ ,  $\gamma$ ,  $\delta$ , and  
 10  $\zeta$  and  $\eta$  chains, are responsible for coupling the ligand binding to signalling pathways that result in T-cell activation and the elaboration of the cellular immune responses. Despite the gene diversity of the TCR chains, two structural features are common to all known subunits. Firstly, they are transmembrane proteins with a single transmembrane spanning  
 15 domain - presumably alpha-helical. Secondly, all the TCR chains have the unusual feature of possessing a charged amino acid within the predicted transmembrane domain. The invariant chains have a single negative charge, conserved between the mouse and human, and the variant chains possess one (TCR- $\beta$ ) or two (TCR- $\alpha$ ) positive charges. Listed below in TABLE 1 is  
 20 the transmembrane sequence of TCR- $\alpha$  in a number of species showing that phylogenetically this region is highly conserved indicating an important functional role. The substitutions between species are very conservative.

TABLE 1. Sequence comparison of TCR- $\alpha$  transmembrane region

25

SPECIES	SEQUENCE
MOUSE	NLSVMGLRILLKLVAGFNLLMTL
RAT	NLSVMGLRILLKLVAGFNLLMTL
SHEEP	NLSVTVFRILLKLVVGFNLLMTL
COW	NLSVI VFRILLKLVVGFNLLMTL
HUMAN	NLSVI GFRILLKLVAGFNLLMTL

Studies on the assembly of the multicomponent TCR by Manolios *et al* (1990, 1991, 1994) showed that the stable interaction between TCR- $\alpha$  and CD3- $\delta$  and TCR- $\alpha$  and CD3- $\epsilon$  was localised to eight amino acids within the

transmembrane domain of TCR-  $\alpha$  (shown above in bold) and it was the charged amino acids arginine and lysine that were critical for this process. This finding exemplified the fact that amino acids within the transmembrane domain not only functioned to anchor proteins but were  
5 important in the assembly of subunit complexes and protein-protein interactions.

The above system depended on the modification of complementary strand DNA (cDNA) to create a number of protein mutants. Chimeric cDNA molecules were transfected into COS (fibroblast line) cells to express the  
10 required protein. Coexpression of these chimeric proteins were used to evaluate the region of interaction. Reiterating the above, the technology involved cDNA manipulation, metabolic labelling, immunoprecipitation and gel electrophoresis.

Transmembrane domains are small in size and proteins transversing  
15 this region are usually constrained to an alpha-helical configuration. These biophysical features coupled with the ability to engineer protein-protein interactions via transmembrane charge groups suggested to the present inventor a possible new approach to intervene and potentially disturb TCR function.

20 The present inventor has developed a series of peptides that are inhibitors of function of this crucial receptor, presumably by interfering with assembly. The present inventor has also found that these peptides have an effect on T-cell mediated inflammation and that carboxyl terminal conjugation did not alter the function of the peptides. This is exemplified by  
25 coupling peptide to a lipid carrier system with increased effect and no loss of function. In addition, the present inventor has also found that the peptide alone had the ability to translocate intracellularly making it a potentially effective drug delivery system. The efficacious clinical manifestations of the administered peptide would be a decrease in inflammation, e.g. as  
30 demonstrated by a decrease of arthritis in an adjuvant model of arthritis.

Accordingly, in a first aspect the present invention consists in a peptide of the following formula:-

A-B-C-D-E in which:

A is absent or 1 or 2 hydrophobic amino acids

35 B is a positively charged amino acid

C is a peptide consisting of 3 to 5 hydrophobic amino acids

D is a positively charged amino acid, and  
E is absent or up to 8 hydrophobic amino acids

In a preferred embodiment of the present invention C is 3 or 4  
hydrophobic amino acids.

5 In a further preferred embodiment of the present invention A is 2  
hydrophobic amino acids and E is 1 to 3, and preferably 1, hydrophobic  
amino acids.

In yet a further embodiment of the present invention B is arginine  
and D is lysine or B is lysine and D is arginine.

10 In yet a further preferred embodiment of the present invention the  
peptide is Gly-Leu-Arg-Ile-Leu-Leu-Leu-Lys-Val, Leu-Lys-Ile-Leu-Leu-Leu-  
Arg-Val, Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val or Phe-Lys-Ile-Leu-Leu-Leu-  
Arg-Val.

In a second aspect the present invention consists in a therapeutic  
15 composition comprising the peptide of the first aspect of the present  
invention and a pharmaceutically acceptable carrier.

In a third aspect the present invention consists in a method of  
treating a subject suffering from a disorder in which T-cells are involved or  
recruited, the method comprising administering to the subject a  
20 therapeutically effective amount of the composition of the second aspect of  
the present invention.

The therapeutic composition may be administered by any  
appropriate route as will be recognised by those skilled in the art. Such  
routes include oral, transdermal, intranasal, parenteral, intraarticular and  
25 intraocular.

In a fourth aspect the present invention consists in a method of  
delivering a chemical moiety to a cell comprising exposing the cell to the  
chemical moiety conjugated to the peptide, preferably to the carboxy  
terminal, as claimed in any one of claims 1 to 10.

30 A non-exhaustive list of disorders in which T-cells are  
involved/recruited include:

Allergic diathesis eg delayed type hypersensitivity, contact  
dermatitis

35 Autoimmune disease eg systemic lupus erythematosus, rheumatoid  
arthritis, multiple sclerosis, diabetes, Guillain-Barre syndrome,  
Hashimotos disease, pernicious anemia



Gastroenterological conditions eg Inflammatory bowel disease,  
Crohn's disease, primary biliary cirrhosis, chronic active hepatitis  
Skin problems eg psoriasis, pemphigus vulgaris  
Infective disease eg AIDS virus, herpes simplex/zoster  
5 Respiratory conditions eg allergic alveolitis,  
Cardiovascular problems eg autoimmune pericarditis  
Organ transplantation  
Inflammatory conditions eg myositis, ankylosing spondylitis.

As used herein the term "subject" is intended to cover both human  
10 and non-human animals.

As will be recognised from the above discussion the peptide of the  
present invention is based on a portion of transmembrane domain of TCR- $\alpha$ .  
The complete murine sequence of this portion is  
NLSVMGLRILLKLVAGFNLLMTLRLWSS, whereas the corresponding human  
15 sequence is NLSVIGFRILLKLVAGFNLLMTL. There is complete sequence  
homology across a range of species in the last 15 amino acids of the TCR-  
alpha chain distal to the sequence upon the peptide of the present invention  
is based (shown in bold). Peptides including these additional 15 residues  
may have activity similar to the peptide of the present invention. The  
20 essential feature is that the peptide includes two positively charged amino  
acids separated by 3 to 5 hydrophobic amino acids. Further, as will be clear  
from the following examples, the peptide of the present invention may be  
modified at the carboxy terminal without loss of activity. Accordingly, it is  
intended that the present invention includes within its scope peptides which  
25 include additional amino acids to the "core" sequence of the peptide of the  
present invention and which affect the T-cell antigen receptor.

As demonstrated in the following examples the peptide of the  
present invention is able to enter cells. Accordingly it is envisaged that,  
apart from its other uses, the peptide of the present invention could be used  
30 as a "carrier" to deliver other therapeutic agents to cells. This could be  
achieved, for example, by conjugating the therapeutic to be delivered into  
the cell to the peptide of the present invention.

As will be readily understood by those skilled in this field  
hydrophobic amino acids are Ala, Val, Leu, Ile, Pro, Phe, Tyr and Met, whilst  
35 positively charged amino acids are Lys, Arg and His.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples.

5    **Example 1    Synthesis of Peptide**

The first step was to synthesise a short hydrophobic peptide corresponding to the predetermined assembly sequence. The amino acid sequence of the competitive peptide is NH<sub>2</sub>-Gly-Leu-Arg-Ile-Leu-Leu-Leu-Lys-Val-OH hereafter referred to as "core peptide". Subsequently a number  
10    of other peptides listed in TABLE 2 were synthesised (>95% purity, by Auspep Australia, Melbourne, Australia) and examined for their effect on T-cell function and inflammation.

TABLE 2. Peptides and their sequence

15

PEPTIDE	SEQUENCE
Core peptide	Gly-Leu-Arg-Ile-Leu-Leu-Leu-Lys-Val-OH
A	Met-Gly-Leu-Arg-Ile-Leu-Leu-Leu-OH
B	Leu-Gly-Ile-Leu-Leu-Leu-Gly-Val-OH
C	Leu-Lys-Ile-Leu-Leu-Leu-Arg-Val-OH
D	Leu-Asp-Ile-Leu-Leu-Leu-Glu-Val-OH
E	Leu-Arg-Ile-Leu-Leu-Leu-Ile-Lys-Val-OH
F	Leu-Arg-Leu-Leu-Leu-Lys-Val-OH

**Example 2    Solubility**

The core peptide and other peptides listed above were noted to be hydrophobic and insoluble in aqueous solutions. A variety of solvents and  
20    carriers were tested. These included ethanol, dimethylsulphoxide (DMSO), dimethyl formamide (DMF), trifluoroacetic acid (TFA), squalane oil (2,6,10,15,19,23-hexamethyltetracosane), and lipid conjugation by addition of palmitic acid to the core peptide via TRIS-conjugation (Whittaker R.G., Bender V.J. 1991) to increase solubility. The preferred solvent was DMSO  
25    and the final concentration used in cell cultures ranged from 0.1% - 0.2%. Concentrations of DMSO greater than 1% was toxic to cells. Stock solutions

of peptide and lipopeptide conjugates were dissolved in DMSO and used in a 1/1000 dilution.

The addition of peptide/lipopeptide in DMSO to aqueous solutions resulted in "fat" or "crystal" globules that settled to the bottom of the tissue culture flask and dissolved poorly. These globules could be seen by phase contrast microscopy, but were less obvious for lipid conjugates.

Core peptide containing C<sup>14</sup>-glycine (C<sup>14</sup>-peptide) was synthesised by Auspep Australia and used to study solubility. C<sup>14</sup>-peptide dissolved/suspended in DMSO was added to a final concentration of 100 µM to T-cell media (RPMI 1640 supplemented with 10% foetal calf serum and 0.3% mercaptoethanol: TCM) and shaken. The media was centrifuged and supernatant filtered through 0.2 µM filter or left unseparated. The total radioactivity in unseparated medium was 20,000 cpm, 1000 cpm after the medium was centrifuged and 500 cpm after the media was filtered. These experiments highlight the insoluble nature of the peptide *in vitro* and suggest that approximately 5% goes into solution.

### Example 3 Entry of peptide into cells

To examine if peptide enters cells, C<sup>14</sup>-peptide was added to a flask of 5x10<sup>6</sup> 2B4.11 cells (T-cell hybridoma specific for cytochrome c) in a final concentration of 100 µM and 0.2% DMSO and incubated overnight. The adherent cells were washed four times with phosphate buffered saline (PBS) in the flask, solubilised with triton-containing buffer and radioactivity counted. The amount of radioactivity in the supernatant was 70,000 cpm and 5000 cpm in the solubilised cells.

In a variation of the above experiment, 2B4.11 cells (7.5x10<sup>4</sup>) were grown in Petri dishes containing 2 ml of TCM and a "Transwell" with 0.4 µM membrane was placed in the Petri dish. C<sup>14</sup>-peptide in a final concentration of 100 µM and 0.1% DMSO were added in the "Transwell" and after 24 hr and 48 hr incubation the counts determined on both sides of the filter and in the cells. Approximately 85% of radioactivity was retained in the "Transwell", 8% in the Petri dish media and 7% within cells. The above experiments demonstrated that peptide was able to enter cells. Considering the low solubility of peptide (5%-10%) all of the available peptide in solution entered the cells (7%).

**Example 4 Intracellular localisation of fluoresceinated peptide in T-cells.**

Experiments suggested that the small percentage of peptide that goes into solution can enter/or be taken up by cells. To confirm this, core peptide covalently linked with fluorescein isothiocyanate (FITC) was added to T-cells and intracellular localisation determined by visualisation using confocal or conventional UV light microscopy.

Fluoresceinated labelled core peptide was prepared as follows: 10.25 mg of core peptide was dissolved in 0.5 ml dimethylformamide (DMF) and 2  $\mu$ M of FITC in 0.5 ml of DMF was added dropwise with stirring, at room temperature. The pH was adjusted to 9 with N-methyl, N,N-diisopropylamine, and the reaction allowed to proceed for 1 hr. Semi-preparative HPLC was then used to separate FITC-peptide from free FITC, using a C-4 column (6ml/min; buffer A, 0.1% TFA; buffer B, 80% acetonitrile, 20% water; 0.1% TFA; linear gradient of 40%-100% B). Fractions were monitored by analytical HPLC and the fractions containing pure fluoresceinated core peptide (FITC-peptide) pooled.

Two flasks of cultured 2B4.11 cells ( $5 \times 10^6$ ) were spun down and resuspended in PBS containing calcium and magnesium. To one flask, FITC dissolved in DMSO was added to a final concentration of 10  $\mu$ M and to the other FITC-peptide 10  $\mu$ M. The final concentration of DMSO in both flasks was 0.1% previously shown to have no effects on T-cells. The cells were incubated at 37°C for 30 min and then examined under the confocal microscope.

The observations can be summarised as follows: (i) FITC and FITC-peptide entered the cells; (ii) free FITC gave brighter fluorescence than FITC-peptide in the cells; (iii) the intracellular staining pattern was not different between the free FITC and FITC-peptide. Nuclear and especially bright nucleolar staining was observed; (iv) conjugation of peptide by FITC did not prevent entry of peptide into cells; (v) there was no "leaching" out of cells of FITC-peptide over a 5 hr period. These experiments demonstrate that FITC-peptide could be taken up by cells and localised intracellularly. In conjunction with experiments previously described showing intracellular uptake by  $C^{14}$ -peptide it is evident that it is the inherent nature of the peptide sequence and not its conjugates (FITC,  $C^{14}$ ) that allows cellular entry.

**Example 5 Tris-fat conjugation of core peptide carboxyl terminal**

The effect of carboxyl conjugation of core peptide, as exemplified by lipid conjugation, on the ability of peptide to competitively inhibit the function of this crucial receptor was investigated. The efficacious clinical manifestations of the administered lipopeptide would be a decrease in inflammation e.g. as demonstrated by a decrease of arthritis in an adjuvant model of arthritis, as would be seen with peptide. In addition to the lipoconjugation of core peptide a number of other lipopeptides were synthesised and used as controls in subsequent experiments. The lipopeptides were synthesized according to the methods set out in Whittaker, R.G., Hayes, P.J., and Bender, V.J. (1993) Peptide Research 6, 125 and Australian Patent No. 649242. The disclosure of these references is incorporated by herein by cross reference

Preparation of Fluorescein Labelled Gly-Leu-Arg-Ile-Leu-Leu-Leu-Lys-Val-Gly-Tris-mono- and tri-palmitates. To a solution of each of the deprotected lipopeptides (15 and 6 mg) in DCM (1 ml) a solution of FITC (4 mg 10  $\mu$ mole) in DMF (500  $\mu$ l) was added with stirring. The apparent pH of the reaction was maintained at 9.0 by the addition of triethyl amine (TEA). The fluorescein-labelled mono, and tri-palmitoyl derivative of the peptide were purified by semi-preparative HPLC (C4 column, System B). The purified compounds were evaporated to dryness and lyophilised from tert. butyalcohol to give the fluorescein labelled peptide monopalmitate ( $R_f^B$ , 7.83) and tripalmitate ( $R_f^B$  9.85) which were tested as described below.

TLC of the fluorescein-labelled lipopeptides (DCM : MeOH, 95:5) showed the absence of free FITC and free Gly-Tris-monopalmitate and Gly-Tris-tripalmitate (used in lipopeptide synthesis) (by ninhydrin staining).

Solid Phase Peptide Synthesis. Gly-Leu-Arg-Ile-Leu-Leu-Leu-Lys-Val (core peptide) and its fully protected form, Boc-Gly-Leu-Arg(PMC)-Ile-Leu-Leu-Leu-Lys(Boc)-Val-OH (and the C14 -labelled peptide) were supplied by Auspep Pty Ltd. Both were synthesised by the FMOC-chemistry in the manual mode.

It will also be readily understood by those skilled in the art that there are a number of well known linkers that can be used to join compounds (such as peptides) with a carboxyl group to an amino group. These include:-

a) a linker with an amino group to the compound and a carboxyl group to the Tris (or amino acid if present) such as an amino acid or antibiotic.

- b) a linker with an amino group to the compound and a sulphonic acid group to the Tris (or amino acid if present) such as 2-aminoethanesulphonic acid (taurine).
- c) a linker with an hydroxyl group to the compound and a carboxyl group to the Tris (or amino acid if present) such as glycolic acid, lactic acid etc.
- d) a linker with an hydroxyl group to the compound and a sulphonic acid group to the Tris (or amino acid if present) such as 2-hydroxyethanesulphonic acid (isethonic acid).
- e) a linker with an hydroxyl group to the compound and a reactive halide group to the Tris (or amino acid if present) such as 2-chloroethanol.
- f) other examples of potentially suitable linkers between a compound with a reactive carboxyl and the amino group of Tris (or amino acid if present) include the compound families exemplified by p-hydroxybenzaldehyde, 2-chloroacetic acid, 1,2- dibromoethane and ethyleneoxide.

Linkers could also contain disulphide groups that would reduce to liberate modified peptide intracellularly.

#### 20 Example 6 Localisation of FITC conjugated lipopeptides in COS cells

Using con-focal microscopy, the ability of FITC-conjugated lipopeptides to enter non-T cells (COS cells-fibroblasts) was examined.

##### Materials:

- Stock concentration in DMSO- Core peptide.Tris.monopalmitate. FITC (MW 1862) 10 mM; core peptide.Tris.dipalmitate.FITC (MW 2334) 10 mM; core peptide.Tris.tripalmitate.FITC (MW 2806) 10 mM; glycine.Tris.monopalmitate.FITC (MW 805) 10 mM; glycine.Tris.tripalmitate.FITC (MW 1286) 10 mM; FITC, (MW 390) 6.4 mM.

##### Method:

- 30 COS cells were grown on coverslips until 80% confluent, washed twice with PBS and incubated with FITC conjugated lipopeptides for 15 min or 2 hr. The final concentration of lipopeptides was 10 $\mu$ M, and 6.4 $\mu$ M for FITC, for each time point respectively. Cells were washed twice with PBS, mounted with PBS/glycerol and examined with confocal microscopy.

**Results:**

Experiments indicated that fluorescein-conjugated lipopeptides can transmigrate across cell membranes and localise to within the cellular cytoplasm, reaching as far as the endoplasmic reticulum (ER), where protein synthesis takes place. The extent of cellular penetration was influenced by the lipid moiety attached to the peptide. Of the lipopeptides the monopalmitate had the greater ability to infiltrate within the fibroblasts and T-cells so far examined (see below). The ER is the best site to try and effect assembly. Once all the chains have assembled and transported to the cell surface it may be much harder to disrupt the receptor at the cell surface membrane. Targetting peptides to the ER is an ideal site to disrupt the TCR complex.

**Example 7 Localisation of FITC conjugated lipopeptides in T-cells**

Using con-focal microscopy, the ability of FITC conjugated lipopeptides to enter T-cells was examined.

**Materials:**

Lipopeptides as above. 2B4.11 T-cell hybridoma cell line.

**Method:**

2B4.11 T-cells were grown in TCM and resuspended in a concentration of  $8 \times 10^5$  cells/ml. Viability >95% using trypan blue. One ml of cells was added to polypropylene tubes and washed twice with PBS. Cells were resuspended in PBS and one microlitre of stock FITC-conjugated lipopeptides added for 30 min. Cells were washed with PBS, mounted with PBS/glycerol, and viewed using confocal microscopy.

**Results:**

Similar to that of COS cells (see above). Results showed that lipopeptides were able to enter T-cells. The lipoconjugation of peptide does not prevent entry of peptides into cells and has the potential use of being used as a carrier vehicle to increase solubility.

**Example 8 Effect of peptides and lipopeptides on TCR assembly and cell surface expression on T-cells using flow cytometry analysis****Materials**

The T-cell hybridoma 2B4.11 which expresses a complete TCR on the cell surface was used as a positive control to assess the effects of

peptides on TCR expression. The cells were grown in TCM. The  $\beta$ -deficient variant 21.2.2 and the  $\beta$ - and  $\zeta$ -deficient cell line 3.12.29, derived by repetitive subcloning of 2B4.11 cells (Sussman et al., 1988) and lacking TCR expression were used as negative controls.

- 5       Peptides tested included core peptide, lipopeptides and a peptide from tumor necrosis factor receptor termed 558 (used as a negative control). The final concentration of each substance used in incubation was 10 $\mu$ M.
- Antibodies

- 10       The following antibodies were used for immuno-precipitation and flow cytometry analysis: Mouse IgG2a monoclonal antibody (MAb) against TCR- $\alpha$  chain of the T-cell hybridoma 2B4 (A2B4-2, Samelson et al., 1983), MAb against 2B4.11 TCR- $\beta$  chain (KJ25), hamster IgG anti-CD3- $\epsilon$  MAb (145-2C11 [2C11], Leo et al., 1987), rabbit anti-CD3- $\epsilon$  polyclonal antiserum raised against purified mouse CD3- $\epsilon$  (127, Minami et al., 1987), anti-CD3- $\delta$
- 15       polyclonal antibody (R9) raised in goat immunized with a COOH-terminal peptide of the mouse CD3- $\delta$  chain (Samelson *et al.*, 1986).

#### Method

- 20       FACS analysis: 1 x 10<sup>6</sup> (2B4.11, 21.2.2) cells were incubated with a number of separate peptides and lipopeptides in a final concentration of 10 $\mu$ M overnight. The cells were then washed with PBS and incubated with 50  $\mu$ l primary antibody (A2B4 or 2C11) for 30 mins at 4°C. Cells were washed twice in PBS and 0.1% BSA and incubated for an additional 30 min at 4°C with FITC-labelled second antibody. Cells were washed two additional times with PBS and 0.1% BSA prior to analysis on a Becton-
- 25       Dickson FACS Analyser or Becton-Dickson FACS Scan.

#### Results

- 30       The expression of TCR on 2B4.11 cells treated with core peptide control peptide, or lipopeptides did not alter the cell surface expression of the receptor. These experiments have been repeated with higher concentration of core peptide (100 $\mu$ M) and longer incubation times ranging from 1-10 days and the results have been the same showing no change in T-cell surface antigen receptor expression.

The following experiments were performed to assess the *in vitro* effects of peptides/lipopeptides on T-cell function.



**Example 9 Antigen presentation assay I**

An antigen presentation assay (described below) examined the ability of a number of peptides to inhibit T-cell activation following antigen recognition, by measuring the product of T-cell activation, Interleukin-2 (IL-2).

5       **Material.** The following cell lines were used: 2B4.11, a T-cell hybridoma that expresses a complete antigen receptor on the cell surface (Samelson et al., 1983) and produces IL-2 following antigen recognition (cytochrome c). Interleukin-2 dependent T- cell line (CTLL) for conventional  
10       biological IL-2 assays; and the B-cell hybridoma cell line LK 35.2 (LK, I-E<sup>k</sup> bearing; Kappler et al., 1982) which acts as the antigen presenting cell. The hybridomas were grown in TCM. Cytochrome c (Sigma, USA) was added in the media to give a final concentration of 50  $\mu$ M in the antigen presenting assay.

15       **Peptides examined** included: core peptide, seven other control peptides from a variety of sources having an equivalent length to core peptide and peptides A, B, C, D, E and F. The final concentration of the peptides in the antigen presentation assay was examined at several levels ranging from 10  $\mu$ M to 100  $\mu$ M.

20       **Method.** For T-cell antigen stimulation  $2 \times 10^4$  LK35.2 cells were co-cultured with 50  $\mu$ M pigeon cytochrome c dissolved in PBS and  $2 \times 10^4$  2B4.11 T-cells for 18 hr. The assay was done in triplicate. Supernatants were recovered and IL-2 content determined by CTLL proliferation. The incorporation of <sup>3</sup>H-thymidine is directly proportional to the amount of IL-2  
25       present in the supernatant. The ability of different peptides to inhibit IL-2 production was examined. In addition to measuring <sup>3</sup>H-thymidine incorporation, IL-2 measurements (IU/ml) were also determined.

30       **Results.** In assays where either cytochrome c (antigen) or LK cells (antigen presenting cells) were omitted there was no IL-2 production. The lack of T-cell activation under such conditions indicated that there was no lipopolysaccharide (LPS) or endotoxin in the solutions which may have non-specifically stimulated the T-cells. The combination of all three constituents of the assay at the concentrations shown above resulted in the production of IL-2 as measured by high <sup>3</sup>H-thymidine incorporation by CTLL cells (22,000  
35       cpm). When core peptide or other analogues were added to the assay system the amount of IL-2 produced varied respectively. All peptides tested at

10  $\mu\text{M}$  had no effect on IL-2 production. The best effect was noted with core peptide at 100 $\mu\text{M}$  and peptide C (100 $\mu\text{M}$ ) leading to a 15%-30% reduction in IL-2 production compared to control. This was reproducible on at least three separate occasions. Peptides A, B, D, E and F had a variable and minor effect on T-cell activation. The seven control peptides with equivalent length but no sequence homology to the peptide had no effect on IL-2 production.

Preincubation of core peptide and other peptides at 37°C in TCM prior to addition in the antigen presenting assay, improved solubility and activity and was reflected as an additional incremental increase above baseline activity noted with freshly prepared peptide.

#### Example 10 Antigen presentation assay II

The following experiments were performed to assess the *in vitro* effects of lipopeptides on T-cell function.

Material. Lipopeptides examined included: core peptide Tris-monopalmitate (100 $\mu\text{M}$  and 0.1% DMSO) and core peptide Tris-tripalmitate (100 $\mu\text{M}$  and 0.2% DMSO). The final concentration of the two lipopeptides in the antigen stimulation assay is shown in brackets respectively.

Method. As described in Example 9.

Results. Initially, when core peptide Tris-tripalmitate (100 $\mu\text{M}$  and 0.2% DMSO) was added, there was a reduction in T-cell activation by 75% (highest count 5,190 cpm of 22,000 cpm for control). The addition of core peptide Tris-monopalmitate (100 $\mu\text{M}$  and 0.1% DMSO) had a profound effect on IL-2 production, with only 137 cpm recorded (similar to background).

The concentration of 0.1% DMSO and 0.2% DMSO used in the test system was examined and not found to influence IL-2 production. Subsequent experiments have confirmed these findings and show an IL-2 reduction of 86%-92% compared to control. Palmitic acid alone (100 $\mu\text{M}$ ) used to conjugate the peptide, added to the antigen presenting assay system did not affect IL-2 production.

The following experiments examined the ability of core peptide to circulate within the animal following administration, and the effects on experimentally induced inflammation.

**Example 11 Distribution of C<sup>14</sup>-core peptide**

To examine the distribution of subcutaneously injected peptide in mice, C<sup>14</sup>-core peptide (5mg/mouse) was dissolved in 150 microlitres of squalane oil and injected at the base of the tail of Balb/c mice. After 24 hr, counts were measured from pulped organs. Distribution of the peptide was noted in thymus (5%), spleen (7%), blood (3%) and a large proportion in lymph nodes (28%), kidney (30%) and liver (28%).

Experiments were extended to examine the ability of core peptide to prevent disease in animal models of inflammation. Three *in vivo* experimental models including adjuvant induced arthritis in rats, cyclophosphamide induced diabetes in NOD mice, and experimental allergic neuritis in rats were used. In these models, encompassing two species, core peptide was able to influence the degree of inflammation.

**Example 12(a) Adjuvant induced arthritis in rats**

The rat adjuvant arthritis model is a classic model of inflammation which has been used extensively by a number of laboratories to study disease progression and effects of potential new anti-inflammatory drugs thereon over the last 30 years (Pearson et al., 1961; Cremer et al., 1990; Holmdahl and Kvick, 1992; Cannon et al., 1993). This model has also been widely used by researchers at the Royal North Shore Hospital over the last 10 years and procedures have been established for the study of this model of inflammation. All procedures on the animals were carried out under halothane/oxygen/nitrous oxide anaesthesia (2%v/v halothane in 1 litre/min O<sub>2</sub> and 2 litres/min N<sub>2</sub>O). Rats were injected intradermally at the base of the tail with a minimal adjuvant dose (1 mg heat killed *Mycobacterium tuberculosis* [MTB] in 100 µl squalane) once and only once. The method for coadministrating test samples with MTB was first described by Whitehouse et al., 1990. At regular intervals between days 0-28, animals were weighed and their arthritic condition assessed by measurement of maximum tail thickness and rear paw thickness (with a micrometer screw gauge). Rats were housed in holding bins after the initial tail injection and allowed access to unlimited water and pellet food. On day 29 the animals were sacrificed.

Materials. The first experiment consisted of 12 rats weighing approximately 190-210 grams that were purchased from the Perth Animal Resource Centre (ARC) and maintained in the Gore Hill Animal House

facility. Used were core peptide (30mg) suspended in adjuvant (0.6 ml squalane containing 7 mg MTB), core peptide Tris-monopalmitate (15mg) suspended in 0.6 ml adjuvant, core peptide Tris-tripalmitate 20 mg/0.6 ml of adjuvant.

5        Rats were divided into four groups, each group containing three rats. First group received adjuvant only (positive control), second group adjuvant with core peptide, third group core peptide.Tris. monopalmitate suspended in adjuvant, and last group core peptide.Tris. tripalmitate in adjuvant. Rats were injected with the above compounds in a 0.1 ml volume at the base of  
10      the tail. Baseline measurements of rat weight, paw width, and tail diameter were made on Day 0, and subsequently on day 4, 7, 9, 14, 16, 18, 21, 25 and 28. Arthritis was graded and animals sacrificed if there was marked swelling, redness and obvious discomfort. Not all rats given MTB developed arthritis. In general more than 80% of control rats developed arthritis.

15       Results. After 18 days all the control animals given adjuvant only had developed arthritis and had to be sacrificed. Two of the three core peptide treated animals (2/3) had no evidence of arthritis. Similarly, two of the three animals given core peptide.Tris.tripalmitate had no evidence of arthritis. Animals given core peptide.Tris.monopalmitate and adjuvant all  
20      developed arthritis. However, the onset and development of arthritis in this latter group was prolonged by 3-4 days and the clinical severity was much reduced (number of joints, paw swelling, loss of weight) compared to controls.

25       Experiments using adjuvant induced arthritis in rats showed that the peptide and its lipid conjugate had a protective effect on the induction of arthritis in this animal model. Results of repeat and subsequent experiments using a number of different peptides (7mg/rat) and drugs are summarised in TABLE 3.

**TABLE 3.** Effects of different peptides on adjuvant induced arthritis in rats.

PEPTIDE	INDUCTION OF ARTHRITIS		EFFECT
	MTB ALONE	WITH PEPTIDE	
CORE	3/3 (100%)	1/3 (33%)	Protective
	3/5 ( 60% )	1/5 (20%)	Protective
	5/5 (100%)	1/4 (25%)	Protective
A	2/4 (50%)	4/6 (67%)	No effect
B	2/4 (50%)	2/4 (50%)	No effect
C	4/5 (80%)	0/4 (0%)	Protective
D	4/5 (80%)	4/5 (80%)	No effect
E	5/5 (100%)	3/5 (60%)	Protective
F	5/5 (100%)	0/5 (0%)	Protective
CS*	5/5 (100%)	1/5 (20%)	Protective
DXM*	5/5 (100%)	4/4 (100%)	No effect+

- 5 CS\*, cyclosporin, 50mg/kg; DXM, dexamethasone (2mg/kg).  
 +, animals developed arthritis but the onset of arthritis was delayed by 3- 4 days.

The results of the above experiments indicated that core peptide had an effect on inflammation both to delay its onset, decrease severity, and prevent onset of disease. These effects were similar to those obtained with the co-administration of cyclosporin and adjuvant. Cyclosporin is a well known and widely used immunosuppressive agent. There was no indiscriminate effect of peptide action. Best results were noted with core peptide, peptide C and F. Core peptide and peptide C each have charged amino acid groups at the same site but the amino acids reversed. This indicated that it was the charge group rather than the particular amino acid that was important. In contrast there was no effect noted with peptide B or D having either no or negative charge group amino acids respectively. Extending the amino acids downstream towards the carboxy terminus had no negative effect. This observation confirms that carboxy modification can be performed without loss of biological activity. Therefore these peptides can be used as carrier peptides for the delivery of other chemical moieties. Increasing the amino acid number between the two polar charge groups

(peptide E) resulted in lower efficacy. Decreasing the number of amino acids between the charged groups (peptide F) had no negative effect.

**Example 12(b)      Dosage effect of core peptide**

- 5            Based on previous experiments by Whitehouse et al (personal communication) an initial dose of 5-7mg/rat was given. To evaluate a lower limit, a number of different core peptide concentrations were examined. The results (TABLE 4) indicated that in addition to a specific action of core peptide it was limited by its effects by dosage.

10

TABLE 4. Effect of core peptide dosage on adjuvant induced arthritis.

PEPTIDE	INDUCTION OF ARTHRITIS		EFFECT OF PEPTIDE
	MTB ALONE	WITH PEPTIDE	
7 mg	11/13 (85%)	3/12 (25%)	Protective
3.5 mg	4/5 (80%)	2/5 (40%)	Protective
1.7mg	6/7 (86%)	7/8 (88%)	No effect

**Example 12(c)      Tail diameter measurements**

- 15            A feature of adjuvant induced arthritis is the development of inflammation in the tail. Tail measurements (mm) between saline injected rats and core peptide treated rats were not statistically significant. Tail diameters from MTB treated rats however were significantly increased ( $p < 0.001$ ) compared to saline and core peptide treated rats ( $p < 0.001$ ),

20    TABLE 5.

TABLE 5. Effect of core peptide on tail inflammation as assessed by tail thickness (mm)

TREATMENT	DAY					
	0	5	10	15	20	25
SALINE (n=4)	7.13	7.75	7.95	8.48	8.78	8.80
MTB only (n=5)	6.86	8.80	8.96	9.46	9.60	9.73
MTB+PEPTIDE (n=5)	7.23	7.62	8.24	8.76	8.96	9.10

- 5 In addition to the oedema noted in the tails, MTB alone given to rats caused ulceration and inflammation at the site of injection that was not present with rats given core peptide or saline.

Example 13 Experimental allergic neuritis (EAN)

- 10 To further confirm the ability of core peptide to delay and diminish the severity of disease induced by T-cells a different model (experimental allergic neuritis) was tested "blind" by independent experimenters (Associate Professor Pollard and Mr J Taylor) at a different institution (University of Sydney).

15 Materials and Methods

- Animals. Male Lewis rats weighing between 239-451 grams were obtained from the Bosch Animal House, University of Sydney colony or from ARC, Perth. All experiments were conducted in accordance with experimental guidelines approved by the Animal Care and Ethics Committee of the University of Sydney.

- 20 Induction of EAN. Lewis rats were immunised in each hind footpad with 50-75 µl of bovine peripheral nerve myelin (PNM) emulsified in complete Freund's adjuvant. The immunisation emulsion consisted of equal volumes of saline and incomplete Freund's adjuvant (Sigma, USA) mixed with bovine PNM and MTB (strain H37RA, DIFCO) added at 15 mg/ml and 5 mg/ml respectively. Where animals received ovalbumin/peptide the peptide was added to the immunisation emulsion at 70mg/ml. These

experiments were performed with the experimenters having no knowledge of what peptide was in the immunisation emulsion.

Animals were observed at least every second day post immunisation for clinical signs and were scored using the following scale: 0, normal; 0.5, weak tail; 1, flaccid tail; 1.5, limp tail and ataxia in hind legs; 2, paraparesis; 2.5, limp tail and severe paraparesis; 3, paraplegia; 3.5, limp tail and paraplegia and forelimb paresis; 4, quadraparesis; 4.5 limp tail and quadraplegia; 5, dead.

Peripheral Nerve Myelin Isolation. Bovine PNM was prepared essentially as described by Norton and Podulso (1973).

#### Results.

A representative example is shown in Figure 1. In this experiment core peptide delayed induction and clinical severity of disease. Similar data were observed for peptide C. These data confirm the efficacy of core peptide and peptide C as general immunosuppressants.

#### Example 14 Diabetes in NOD/Lt (F) mice

In yet another model of T-cell mediated disease the effects of subcutaneously injected core peptide on the induction of diabetes in NOD/Lt (F) mice was tested "blind" by an independent experimenter (Prof L Harrison, WEHI, Melbourne).

A cellular autoimmune process that selectively destroys the pancreatic islet beta cells is thought to be responsible for the development of insulin-dependent diabetes mellitus (IDDM) in humans and in the spontaneous animal models including the NOD mouse (Leiter et al., 1987). A common histopathological feature associated with the development of IDDM is insulitis, the presence within and around the islets of mononuclear cells consisting predominantly of T lymphocytes and to a lesser extent macrophages (Foulis et al., 1986). Experimental strategies aimed at suppressing cellular autoimmunity such as neonatal thymectomy, administration of cyclosporin A or administration of anti-T lymphocyte antibodies prevent the development of diabetes (Campbell et al., 1991).

#### Animals

NOD/Lt (F) mice 10 weeks old at experimental Day 0. This is a high-incidence strain commonly used as an animal model for diabetes.



### Materials

Core peptide dissolved in squalane at a stock concentration of 3.33 mg/ml. Ovalbumin used as a control was suspended in squalane at a stock concentration of 3.33 mg/ml. Peptide and ovalbumin were "solubilised" just prior to injection, with vortexing. A total of 250 µg (75 µl) was injected subcutaneously on the right flank on Day -1, 0 and 1. Mice were given intraperitoneal cyclophosphamide in water at 300 mg/kg on Day 0. Blood glucose measurements were taken on Day 0, 10, 14 and 21. There were 16 mice in the treatment group and 16 in the ovalbumin control group.

Results. Experiments demonstrate a protective effect of core peptide on the induction of autoimmune beta cell destruction which manifests as diabetes (TABLE 6). This finding again confirms the general immunosuppressive ability of core peptide in a different T-cell mediated disease model.

TABLE 6. Effects of core peptide on the induction of diabetes in NOD/Lt (F) mice

TREATMENT	PERCENTAGE OF MICE DEVELOPING DIABETES			
	DAY 0	DAY10	DAY14	DAY 21
Ovalbumin (n=16)	0%	0%	42%	65%
Peptide (n=16)	0%	0%	5%	12%

### Summary

In recent years a vast number of different methods have been used and devised to interfere with the interaction between TCR, MHC or antigen (trimolecular complex) and thereby influence immune responses. The therapeutic potential associated with the development of these ideas and methods of application has not been overlooked. The strategies have included the use of monoclonal antibodies to block MHC or TCR interactions, blocking antibodies to important co-stimulatory or regulatory proteins on the T-cell surface, vaccination with disease inducing T-cells, or TCR epitopes, competing antigens, and inhibition of cytokines or their

receptors. The ability to disrupt TCR function by a specific competitive peptide designed to affect assembly has not been previously reported and examined. The present inventor has clearly shown that the peptides of the present invention are able to inhibit T-cell mediated immune responses in at least three different models by a mechanism previously unreported.

The experiments described herein were principally conducted with a core peptide deliberately chosen to be homologous with the known sequence of the mouse and rat TCR alpha chain. However, for clinical treatment of humans a peptide containing a phenylalanine residue instead of leucine towards the amino terminus of the core peptide may be preferable to maximise homology with the known human sequence (TABLE 1).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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**CLAIMS:-**

1. A peptide of the following formula:-  
A-B-C-D-E- in which:  
A is absent or 1 or 2 hydrophobic amino acids  
5 B is a positively charged amino acid  
C is a peptide consisting of 3 to 5 hydrophobic amino acids  
D is a positively charged amino acid, and  
E is absent or up to 8 hydrophobic amino acids
2. A peptide as claimed in claim 1 in which C is 4 hydrophobic amino  
10 acids.
3. A peptide as claimed in claim 1 in which C is 3 hydrophobic amino  
acids.
4. A peptide as claimed in any one of claims 1 to 3 in which A is 2  
hydrophobic amino acids.
- 15 5. A peptide as claimed in any one of claims 1 to 4 in which E is 1 to 3,  
preferably 1, hydrophobic amino acids.
6. A peptide as claimed in any one of claims 1 to 5 in which B is  
arginine and D is lysine or B is lysine and D is arginine.
7. A peptide as claimed in claim 1 in which the peptide is  
20 Gly-Leu-Arg-Ile-Leu-Leu-Lys-Val.
8. A peptide as claimed in claim 1 in which the peptide is  
Gly-Phe-Arg-Ile-Leu-Leu-Lys-Val.
9. A peptide as claimed in claim 1 in which the peptide is  
Leu-Lys-Ile-Leu-Leu-Leu-Arg-Val.
- 25 10. A peptide as claimed in claim 1 in which the peptide is  
Phe-Lys-Ile-Leu-Leu-Leu-Arg-Val.
11. A therapeutic composition comprising the peptide as claimed in any  
one of claims 1 to 10 and a pharmaceutically acceptable carrier.
12. A method of treating a subject suffering from a disorder in which T-  
30 cells are involved or recruited, the method comprising administering to the  
subject a therapeutically effective amount of the therapeutic composition as  
claimed in claim 11.
13. A method of delivering a chemical moiety to a cell comprising  
exposing the cell to the chemical moiety conjugated to the peptide as  
35 claimed in any one of claims 1 to 10.

1/2

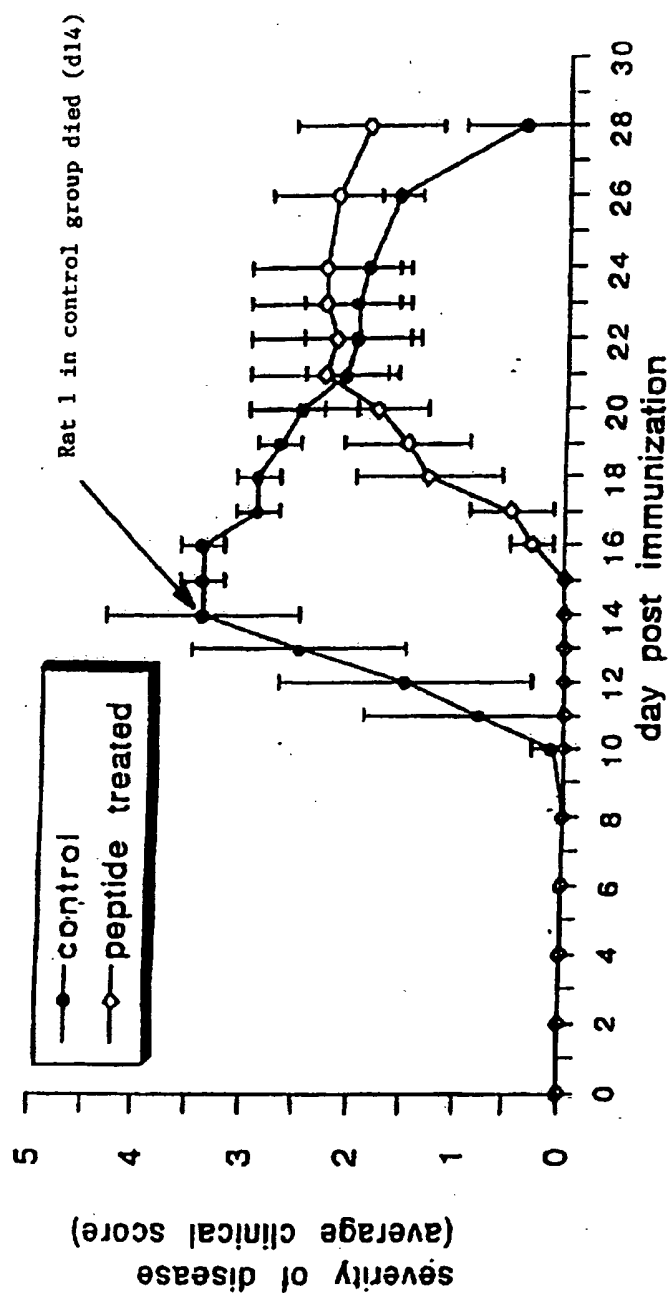


FIGURE 1A

2/2

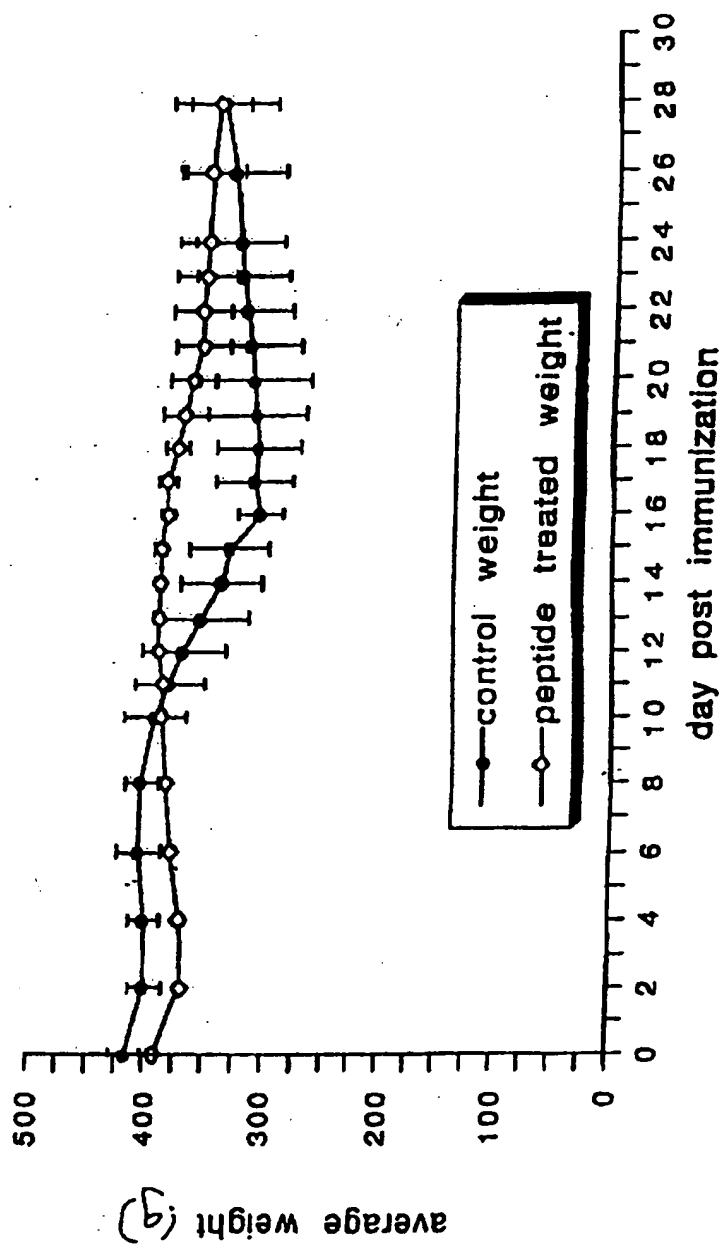


FIGURE 1B

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 96/00018

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : C07K 7/06, 7/08, A61K 38/08, 38/10		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) <b>CHEMICAL ABSTRACTS</b>		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) <b>CHEMICAL ABSTRACTS KEY WORDS: see attached annex A.</b>		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	WO, A, 95/25122 (The Scripps Research Institute) published 21 September 1995., C07K 14/18, A61K 38/04. (see especially page 45, sequence 1D 15)	1-13
<input type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 20 March 1996		Date of mailing of the international search report 27 March 1996 (27.03.96)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929		Authorized officer  <b>CARMELA MONGER</b> Telephone No.: (06) 283 2486



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00018

Box

FIELDS SEARCHED:

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